Hepatitis C Virus Genotypes in French Haemophiliacs: Kinetics and Reappraisal of Mixed Infections

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The distribution and kinetics of hepatitis C virus (HCV) genotypes and the prevalence of mixed infections were studied in a group of 45 French patients with haemophilia A or B or von Willebrand's disease, 21 of them being anti-human immunodeficiency virus (HIV) positive; genotyping was carried out by three methods based on the core, 5′ untranslated region (5′UTR), and the detection of type-specific NS4 antibodies. Genotyping of the 5′UTR revealed genotypes 1a (n = 10), 1b (n = 13), 2a (n = 3), 2b (n = 4), 2NC (n = 3), 3a (n = 10), and two mixed infections (1a + 1b and 3a + 2). Five of 33 patients showed a change from one HCV genotype to another. The core genotyping assay showed 8 of 45 mixed infections: 6/8 1a + 1b and 2/8 3a + 2. Sequencing of core polymerase chain reaction (PCR) products showed that mixed infection 1a + 1b could be explained by nonspecific annealing of the 1b primer to type 1a sequence. By designing new primers whose sequence was more specific to HCV types 1a and 1b, we could confirm 1a + 1b mixed infection in only one of six cases. Serotyping assay showed for 17 of 21 anti-HIV negative patients a concordance with the 5′UTR genotype; however, only 6 of 19 anti-HIV positive patients showed detectable serological reactivity. In summary, we have observed a similar HCV genotype distribution between our haemophilic group and the French anti-HCV positive patients. The study demonstrates the difficulties of assessing with the presently available genotyping and serotyping assays the real prevalence of mixed infections in multiply transfused patients. ***J Med Virol 51:36–41, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: HCV; HCV genotypes; mixed infections; haemophilia; HCV serotypes

INTRODUCTION

Hepatitis C virus (HCV) is a positive-stranded RNA virus of 9,400 nucleotides [Choo et al., 1989] with homology to the flavivirus family. The genome includes a 5′ untranslated region (5′ UTR) [Bukh et al., 1992], a long sequence encoding for a polyprotein processed secondarily in structural (core and envelopes [E1-E2]) and non-structural (NS2-NS5) proteins and a 3′ UTR of variable length [Choo et al., 1991; Takamizawa et al., 1991; Matsuura and Miyamura, 1993]. The 5′UTR and core are highly and well-conserved sequences. In contrast, the putative envelope genes revealed much more significant divergence: in particular, "hypervariable" regions at the N-terminal part of E2 have been demonstrated [Higashi et al., 1993; Okamoto et al., 1991].

The HCV genome shows significant variability which was proven by comparative analyses of the numerous full-length and partial sequences of various isolates in distinct geographical areas. Although there is no final agreed classification of genotypes, it is generally accepted that there are six major genotypes and numerous subtypes [Bukh et al., 1994; Simmonds et al., 1994a,b]. Several studies have shown a correlation between HCV genotypes and the rate of response to interferon (IFN) treatment [Yoshioka et al., 1992; Kanai et al., 1992]. In addition, there is evidence that HCV type 1b might be associated with more severe liver diseases, but this is still a debated issue [Pozzato et al., 1992; Feray et al., 1992, 1993; Nousbaum et al., 1995].

HCV is now the most common cause of post-transfusion and sporadic non-A/non-B hepatitis (NANBH). The introduction of clotting factor concentrates in the 1970s is largely responsible for the transmission of HCV in hae-

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mophilic populations. At the present, most haemophiliacs who received non-virus-inactivated concentrates show evidence of past or present HCV infection. This view is strengthened by data showing a high prevalence (49–100%) of antibodies against HCV (anti-HCV) in haemophilic populations [Rumi et al., 1990; Ludlam et al., 1989]. In French haemophiliacs who have been transfused repeatedly, anti-HCV antibodies are detected in 69.6% of patients, 68% of these showing detectable viraemia [Pol et al., 1994]. Long-term therapy with non-virus-inactivated concentrates may also lead to reinfection and thus coinfection by different HCV genotypes. There is indeed now solid evidence, from several studies in both infected humans and chimpanzees, for superinfection with heterotypic or even homotypic HCV isolates [Lai et al., 1994; Okamoto et al., 1994]. However, only a few studies have so far focused on the dynamics of HCV genotypes and on detailed investigation of mixed infections in haemophiliacs [Jarvis et al., 1994, 1995].

On this basis, we describe the distribution and dynamics of HCV genotypes in a population of French haemophiliacs, by means of two different molecular genotyping assays in the core and 5′UTR and a serotyping test.

PATIENTS AND METHODS

Patients

The 260 haemophiliacs from our Haemophilic centre comprise 69% anti-HCV positive haemophiliacs. We analysed 45 patients affected by haemophilia A or B and von Willebrand's disease (37, 7, and 1, respectively). The sera of these patients were known to be positive to anti-HCV (second-generation assays), 21 were also positive for anti-HIV (15 of 21 and 5 of 21 affected by haemophilia A and B, respectively, and 1 of 21 by von Willebrand's disease). Twenty-eight patients (62%) had been transfused repeatedly (>500 U/kg/year). Thirty-four patients had been transfused with non-virally inactivated concentrates before 1985; all patients received heat-treated factor plasma derivates between 1985 and 1987; since 1987, all patients have received solvent-detergent-treated factors. Most B haemophiliacs were exposed to concentrates prepared from plasma pools coming from volunteer French blood donors, whereas patients with A haemophilia received both French and foreign products. No patient was treated by alpha-IFN. Histological examination of liver biopsies was available for 6 of 45 patients; all six cases have chronic active hepatitis (CAH) [Pol et al., 1994]. Serial serum samples were studied in 36 of the 45 patients (min:56; max:171). Sera from all subjects were stored under frozen conditions (−80°C) until used for the study.

METHODS

RNA Extraction

Viral RNA was extracted from 100 μl of serum by the acid phenol guanidium thiocyanate-modified method [Chomczynsky and Sacchi, 1987] (RNAzol B, Bioprobe System). After chloroform extraction, RNA was precipitated with isopropanol. The pellet was washed with 70% ethanol and resuspended in 30 μl of DEPC water with 40 U of RNAsin (GIBCO-BRL).

Nested PCR and Genotyping Assays

5′ UTR. Ten microlitres of RNA solution were denatured at 65°C for 10 min with 10 pmol of downstream primer [Stuyver et al., 1993]; cDNA synthesis was carried out at 37°C for 45 min with 100 U of MMLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) in 30 μl reaction volume containing 50 mM Tris-HCl, 75 mM KCl, 6 mM $MgCl_2$, 1 mM NTPs. cDNA was stored at −20°C. One fifth of the cDNA was subjected to nested PCR in a single tube [Giannini et al., 1995] with a lower mix containing 5 pmol of each biotinylated external primers in the presence of 5 U of Taq DNA polymerase (P. Elmer, Cetus, Norwalk, CT); the lower mix was thus covered with 200 μl of high-density oil and the upper mix containing 80 pmol each of biotinylated internal primers [Stuyver et al., 1993] was injected onto the surface of the oil. After a first amplification of 30 cycles, the lower and upper mixes were homogenised by centrifugation for 1 min; then a second amplification of 30 cycles was carried out [Giannini et al., 1995]. Furthermore, carry-over prevention was undertaken as described by Kwok and Higuchi [1989]. In addition, dUTP was incorporated instead of dTTP and 1 U of uracil-DNA-glycosylase (UDG, Boehringer Mannheim) was added to the lower and upper mixes, to eliminate PCR contaminations from previous PCR reactions. The PCR products were subjected to electrophoresis on a 2% agarose gel and the positive products were genotyped with InnoLiPA procedure in accordance with the manufacturer's instructions as described previously [Giannini et al., 1995; Stuyver et al., 1993]. The strips were analysed and the genotype assigned according to the pattern of PCR product hybridisation.

Core region. The cDNA was synthetized with primer 186 and amplified by nested PCR in a single tube, as described previously [Nousbaum et al., 1995; Giannini et al., 1995]. Core HCV typing was carried out by a modified method. Thus, a different nested PCR in a single tube was carried out for each genotype using the genotype-specific antisense primer to increase the specificity of the method [Okamoto et al., 1992; Nousbaum et al., 1995]. For the detection of the European genotype 2 we used CRIIIa antisense primer [Giannini et al., 1995]. The nested PCR products were subjected to electrophoresis on 14% acrylamide/bisacrylamide gel, and subtypes were determined by differences in electrophoretic mobility.

To ensure the specificity of the results concerning types 1a and 1b, new primers, whose sequences were more specific than our isolates, were synthetized. The first PCR was carried out for 28 cycles with outer primers CAP1 (295 5′ ATAGGGTGCTTGCGAGTGCCC 3′ 316) and CAP5 antisense (568 5′CCAAGGGTACCCGGGCTGAG 3′ 588) with the following reaction cycle: 94°C for 45 sec, 58°C for 45 sec, and 72°C for 45 sec. The second PCR was thus carried out for 30 cycles at the same conditions using a universal biotinylated sense

primer CAP2biot (5′ 320 GAGGTCTCGTAGACCGTGCA 3′ 340) and one of the two genotype-specific primers: cap1a antisense (5′ 517 5′GGATAGGCTGACGTCTACCT 3′ 537) for genotype 1a and cap1b antisense (5′ 517 5′GGATAGGTTGTCGCCTTCCA 3′537) for genotype 1b. One tenth of the amplified products was electrophoresed on a 2% agarose gel and identified by staining with ethidium bromide (217 bp).

Sequencing of PCR Products

A 5′ biotinylated downstream primer was used for nested PCR. The strands were separated with Dynabeads M-280. The single-strand DNA fragment bound to Dynabeads was used for dideoxy chain termination sequencing with T7 DNA polymerase [Giannini et al., 1995].

Serological Genotyping

Serotyping was carried out by Murex HCV 1,2,3 assay in accordance with the manufacturer's instructions. The test is based on the use of synthetic peptides derived from the NS4 variable antigenic region of HCV types 1,2,3 as capture antigens. The results were analysed and the serotype assigned according to the cut-off calculation [Simmonds et al., 1993].

RESULTS

HCV Genotype Prevalence

The combination of 5′UTR and core-based assays allowed the classification of all 45 patients (Table I). We found genotypes 1(n = 23, 51%; 1a:n = 11, 24.4%; 1b:n = 12, 26.6%); 2(n = 10, 22%; 2a:n = 3; 2b:n = 4; 2NC:n = 3); 3a(n = 10, 22%); and two mixed infections. There was no statistically significant difference among HIV positive and negative individuals. Detailed results obtained with the two approaches are shown in Table I. Eleven of 45 samples showed a discordant result: in six patients classification was observed with the LiPA assay and not with the core-based PCR test. In five additional cases, classification was obtained with both assays, but results were discordant: two of five were 1a in core and 2 in 5′UTR (patients 14 and 16); two of four were 1b in core and 2 in 5′UTR (patients 22 and 33). The remaining patient (no. 8) showed a type 1a with type-specific PCR and a type 1b in 5′UTR. In our experience, such discrepancies generally reflect artefacts in the core-based assay [Giannini et al., 1995].

We also investigated whether reinfection with or reactivation of different genotypes occurred during the period of exposure. As illustrated in Table I, serum samples from 36 (80%) haemophiliacs were analysed twice; those who were PCR positive (n = 33) were genotyped: 28 (84.8%) haemophiliacs were infected with the same genotype at the two time points and 5 patients (15%) showed a change from one HCV genotype to another: 2 from 1a to 1b, 1 from 1b to 1a, 1 from 1a to 2a, and 1 from 1b to 3a.

Sequence analysis in 5′UTR confirmed LiPA genotyping results in all three samples tested (patients 1, 15, and 39). These five patients all had haemophilia A and were multitransfused (except for patient 39); patient 8 developed an inhibitor some months after the first sampling and then received Autoplex ^{R} (American products). Patient 15 was treated with American and Austrian products 4 months after first sampling. Patient 39 received cryoprecipitate once in his life, 5 months before the first sampling. Patient 1 was treated with Autoplex ^{R} for several years and patient 29 was treated with French products obtained from French and foreign blood donors.

Mixed Infections

An additional feature of this study was a detailed analysis of apparently mixed infections. As shown in Table II, discordance was observed between core and LiPA assays. The core genotyping assay showed 8 of 45 mixed infections: six were 1a + 1b and two were 3a + 2; however, it should be noted that only two of the eight mixed infections were confirmed by using the LiPA assay: 1a + 1b and 3a + 2.

There have been previous reports describing a high rate of discordant results concerning mixed infections associated with type 1b with core-based assays [Andonov and Chaudhary, 1994]. Therefore, we reanalysed the products of core-specific PCR from six different cases. Sequence analysis showed that mixed infections 1a + 1b were in fact explained by non-specific annealing of the 1b primer to the type 1a sequence. We synthetised new primers, more specific for HCV types 1a and 1b, to avoid this non-specific annealing. As shown in Table II, when using these primers, 1a + 1b mixed infection was only confirmed in one of six cases. Sequencing analysis further confirmed that only this single sample did indeed contain both 1a and 1b RNA sequences (data not shown).

Serotyping

To investigate further HCV genotypes, we carried out serotyping analysis of 43 serum samples obtained from these 45 subjects at the end of follow-up. Sera were examined by an enzyme-linked immunosorbent assay (ELISA) for a type-specific antibody (Murex 1-3 assay). Because the serotyping assay does not allow distinction among HCV subtypes, only major types of HCV were analysed. As shown in Table III, 21 of the 24 HIV-negative patients showed detectable type-specific antibodies to NS4 peptides. Seventeen of the 21 showed concordance with the 5′UTR genotype and three showed a discrepant result: one was genotype 1b but serotype 3 and two were genotype 2 but serotype 1. For 2 of these 21 subjects, serotype analysis showed evidence for reactivity toward two distinct types: 3 + 1 and 3 + 2; however, PCR-based genotype only revealed one type in these cases: 3 and 2, respectively. Only 6 of 19 HIV positive patients showed detectable serological reactivity. One of these six showed a discordant result: serotype 1 and genotype 3a. One of the five concordant samples had a reactivity for an additional serotype (1 + 3).

DISCUSSION

This study provides a detailed analysis of the HCV genotype distribution in HCV-infected French haemophiliacs. A first point of interest was the similarity of

TABLE I. Distribution and Dynamics of HCV Genotype Analysed in Core and 5'UTR Among 45 Patients Affected by Haemophilia A or B or von Willebrand disease (vWD)*

	Type of haemophilia	Severity of haemophilia	First sample		Follow-up (months)	Type of inactivation	Second sample	
			Core	5'UTR			Core	5'UTR
HIV negative patients								
1	A	Severe	1b	1b	56	I	1a	1a
2	A	Severe	1a	1a	113	I	1a	1a
3	B	Mild	1a	1a	72	II	1a	1a
4	A	Severe	1a	1a	84	III	1a	1a
5	A	Severe	1a	1a	111	I	1a	1a
6	A	Mild	1b	1b	116	I	1b	1b
7	A	Severe	1b	1b	68	III	1b	1b
8	A	Severe	1a	1a	79	III	1a	1a
9	A	Mild	1b	1b	77	II	1b	1b
10	A	Severe	1b	1b	92	I	NC	1b
11	A	Severe	1b	1b	102	II	NC	1b
12	B	Mild	1b	1b	126	I	1b	1b
13	A	Severe	1b	1b	107	I	1b	1b
14	A	Moderate	1a	2	77	III	1a	2b
15	A	Severe	NC	1a	102	I	NC	2a
16	A	Severe	*	*	83	I	1a	2b
17	A	Severe	*	*	106	I	2	2b
18	A	Moderate	NC	3a	87	II	3a	3a
19	A	Severe	3a	3a	66	III	NC	3a
20	A	Mild	NA	NA	/	/	1a + 1b	1a + 1b
21	A	Mild	NA	NA	/	/	1b	1b
22	A	Severe	NA	NA	/	/	1b	2NC
23	A	Mild	NA	NA	/	/	2	2b
24	vWD	Mild	NA	NA	/	/	3a	3a
25	A	Severe	1a	1a	102	I	1a	1a
26	A	Severe	1b	1a	112	I	1a	1a
27	B	Severe	1a	1a	125	I	1a	1a
28	A	Severe	1a	1a	113	I	1a	1a
29	A	Severe	1b	1b	83	I	1b	1b
30	A	Severe	1b	1b	103	I	1b	1b
31	A	Severe	2	2	84	II	2	2a
32	A	Severe	*	*	118	I	NC	2b
33	B	Severe	1b	2	171	I	1b	2NC
34	A	Severe	2	2	116	I	2	2a
35	A	Severe	3a	3a	83	I	2 + 3a	2NC + 3a
36	B	Mild	3a	3a	90	II	3a	3a
37	A	Severe	3a	3a	83	II	3a	3a
38	A	Severe	3a	3a	117	I	3a	3a
39	A	Severe	NC	1b	114	I	3a	3a
40	A	Severe	3a	3a	111	I	NC	3a
41	B	Severe	NC	3a	92	I	3a	3a
42	A	Severe	NA	NA	/	/	1a	1a
43	A	Severe	NA	NA	/	/	1a	1a
44	A	Moderate	NA	NA	/	/	1b	1b
45	A	Moderate	NA	NA	/	/	3a	3a

*NA, not available; NC, not classified; I, patients treated with nonvirally inactivated concentrates; II, patients treated with heat-treated products; III, patients treated with solvent detergent-treated products during the follow-up period. *Asterisks, negative PCR.

the genotype distribution between our haemophilic group and the French anti-HCV positive patients, despite the use of both French and foreign products. Indeed, we found a majority of genotypes 1 and 3 which are the genotypes most represented in France [Nousbaum et al., 1995]. These data are consistent with studies carried out among English haemophiliacs who showed a similar genotype distribution to that of Scottish blood donors from whom the infected blood products were manufactured [Jarvis et al., 1994]. They contrast, however, with subsequent analyses in haemophiliacs from the United Kingdom who received non-viral-inactivated concentrate factors derived from volunteer donors or from commercial products [Preston et al., 1995]. The reasons for these discrepancies are presently unclear. However, in France, the distribution of HCV genotypes among haemophiliacs confirms previous data describing the recent introduction of type 3a due to intravenous drug injections [Pol et al., 1995].

A second and debated issue relates to mixed HCV infections. On the one hand, there are several reports which indicate a relatively high, although very variable, prevalence of such mixed infections in some populations such as haemophiliacs, haemodialysed patients, and intravenous drug users. In particular, figures of 1.6% [Telfer et al., 1995] to 31% [Isobe et al., 1995] have been reported in multitransfused haemophiliacs. There are some potential technical artefacts, especially when us-

TABLE II. HCV Genotype Analysis of Haemophiliacs With Mixed Infections as Revealed by PCR in the Core Region and 5′UTR and Core Sequencing*

Patient no.	PCR				Sequence[a] (core)
	Core			5′UTR	
	1	2	3		
20	1a + 1b	+	+	1a + 1b	1a + 1b
43	1a + 1b	+	−	1a	1a
26	1a + 1b	+	−	1a	1a
8	1a + 1b	+	−	1b	1a
3	1a + 1b	+	−	1a	1a
1	1a + 1b	+	−	1a	1a
35	2 + 3a	NA	NA	2nc + 3a	NA
45	2 + 3a	NA	NA	3a	NA

*1–3 = References of genotype-specific primers (+, sense; −, antisense) in the core region:

1	1st PCR		Nested PCR		2		3	
	+	−	+	−	+	−	+	−
1a	256	186	104	296	CAP1	CAP5	CAP1	CAP5
1b	256	186	104	133	CAP2	CAP1a	CAP2	CAP1b
2	256	186	104	CRIIIA				
3a	256V	186	104V	339				

[a]Direct sequence of PCR products; NA, not available.

TABLE III. Comparison Between HCV Genotyping and Serotyping Results

Genotyping 5′UTR	Serotyping														
	HIV negative[a]								HIV positive[a]							
	1	2	3	NTS[a]	NT[b]	3 + 1	3 + 2	Total	1	2	3	NTS[a]	NT[b]	1 + 3	NA[c]	Total
1a	5							5	2			1	1	1	1	6
1b	6		1		2			9					2		1	3
2a					1			1					2			2
2b	1	2					1	4					1			1
2NC	1							1				1				1
3a			2			1		3	1		2	1	3			7
Mixed infections																
1a + 1b	1							1								
2 + 3a													1			1
Total	14	2	3		3	1	1	24								21

[a]NTS = no type-specific antibody detected
[b]NT = non-reactive
[c]NA = not available

ing core-specific primers, which have been emphasized and might lead to overestimation of the actual prevalence [Andonov and Chaudhary, 1994]. With this view, we undertook a more detailed analysis of such cases: we identified 8 mixed infections among the 45 cases analyzed by using the core genotyping assay; the analysis of these 8 cases by the LiPA assay showed evidence for a mixed infection in only 2 of them. Furthermore, we designed new core-specific primers whose sequences were chosen to provide better discrimination between 1a and 1b; when using these primers, we found no further evidence for 1a + 1b mixed infections in five of six cases; this observation stresses the risk of false positive results for mixed infections with PCR-based assays. This was confirmed by sequence analysis of PCR products for six cases: direct sequencing failed to find evidence of mixed infections in five of six cases and confirmed that only one patient was indeed infected by a mixture of 1a + 1b; interestingly this case was one of the two samples identified by LiPA assay as a mixed infection.

It should also be noted that, when using an assay based on the detection of type-specific NS4 antibodies, we found reactivity to two different HCV types in only three cases. Our investigation therefore does not provide evidence for a high rate of mixed infection in our population of haemophiliacs.

Another important issue addressed in this study was the dynamics of the HCV genotype evolution. We have shown, after a mean follow-up period of 8 years, a change of genotype in 3 of 33 subjects. This rate of HCV type modification is lower than previous reports in haemophiliacs transfused with commercial blood products [Jarvis et al., 1995]. The HCV type change may reflect either reinfection by a different HCV type or reactivation of a previous infection. It should be noted, however, that none of these five cases showed any evidence of mixed infection with a PCR-based assay. One could hypothesize that reinfection or reactivation would be associated with a significant decrease of the replication of the previous HCV type. This observation compli-

cates further the interpretation of the data concerning the rate of detection of mixed HCV infections which might be only identified by testing liver biopsy samples.

In summary, this study shows that in French haemophiliacs, the distribution of HCV genotypes reflects that of the general population and does not evolve frequently with the length of follow-up. It also illustrates that the current assays are not suitable for a true appraisal of mixed HCV infections.

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